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1. Dash et al, Molecular Brain Research 39(1-2) 1996 43-51

2. Nguyen et al Science Aug 19, 1994 265(5175) p1104-7

3. Alberini et al, Assn N Y Acad Sci Jun 30 1995 758 pages 261-86

4. Alberini et al, Cell, March 25, 1994, 76(6) 1099-14

5. Kaang et al Neuron March 1993 10(3) pages 427-35

6. Dash et al, Nature Jun 21 1990, 345 (6277) pages 718-21

7. Bergold et al PNAS 1990 87/10 pages 3788-3791

8. Olds et al New Biol 1991 3/1 pages 27-35 ISSN 1043-4674

9. Brockeroff et al FASEB J 4(4) 1990 A899

10. Thank you!

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C/EBP Is an Immediate-Early Gene Required for the Consolidation of Long-Term Facilitation in Aplysia

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Summary

The consolidation of long-term memory requires protein and mRNA synthesis. A similar requirement has been demonstrated for learning-related synaptic plasticity in the gill-withdrawal reflex of Aplysia. The monosynaptic component of this reflex can be reconstituted in vitro, where it undergoes both short- and long-term increases in synaptic strength in response to serotonin (5-HT), a neurotransmitter released during behavioral sensitization, a simple form of learning. As with sensitization, the long-term synaptic modification is characterized by a brief consolidation period during which gene expression is required. We find that during this phase, the transcription factor Aplysia CCAAT enhancer-binding protein (ApC/EBP) is induced rapidly by 5-HT and by cAMP, even in the presence of protein synthesis inhibitors. Blocking the function of ApC/EBP blocks long-term facilitation selectively without affecting the short-term process. These data indicate that cAMP-inducible immediate-early genes have an essential role in the consolidation of stable long-term synaptic plasticity in Aplysia.

Introduction

Study of various forms of memory storage in humans as well as in simple experimental animals has revealed two characteristic features. First, memory storage has two forms, a short-term form lasting seconds to minutes, and a long-term form lasting days to weeks and sometimes even the lifetime of the organism. Second, long-term memory storage has an initial consolidation phase that is labile and highly sensitive to disruption.

Some of the steps that underlie these two features have been delineated in the gill-withdrawal reflex of the invertebrate Aplysia (Montarolo et al., 1986; Barzilai et al., 1989; Bailey et al., 1992). This reflex can be modified by the four simple forms of procedural learning: habituation, sensitization, classical conditioning, and operant conditioning (Hawkins et al., 1987; Byrne, 1987; Carew and Sahley, 1986). The molecular mechanisms contributing to memory storage have been most extensively studied in the case of sensitization (Bailey and Kandel, 1993). Sensitization is a nonassociative form of learning in which an animal learns about the properties of a single class of stimuli, usually aversive stimuli. When presented with a noxious stimulus to the tail, the animal recognizes the stimulus as aversive and learns to enhance its reflex responses to the siphon stimulus. The duration of the ensuing memory for this noxious stimulus is a function of the number of stimuli applied to the tail (Frost et al., 1985). A single noxious stimulus to the tail produces a short-term memory. The resulting enhancement of the withdrawal reflex lasts for minutes and does not require new protein synthesis. By contrast, four or five noxious stimuli to the tail, separated from each other by 20 min, produce a long-term memory which lasts 1–2 days and requires new protein synthesis (Castellucci et al., 1989). Further training leads to an even more enduring memory reflected in reflex enhancement lasting several weeks (Frost et al., 1985).

Many of the cells that contribute to this reflex have been identified. Of these, the direct (monosynaptic) connections between the siphon sensory neurons and gill motor neurons have been studied most extensively because they are storage sites for both the short- and long-term forms of memory (Montarolo et al., 1986; Schacher et al., 1988; Bailey and Chen, 1983; Frost et al., 1985). Following sensitization training, this set of connections undergoes an increase in synaptic effectiveness, the amplitude and duration of which are functions of the number of training stimuli to the tail. This component of the reflex can be reconstituted in cell culture by plating a single sensory cell with a single motor cell (Montarolo et al., 1986). The two cells form connections in culture that undergo an increase in effectiveness as a function of the number of applications of serotonin (5-hydroxytryptamine, 5-HT]), a modulatory transmitter released in vivo by sensitizing tail stimuli (Clark and Kandel, 1984; Frost et al., 1985; Montarolo et al., 1986; Rayport and Schacher, 1986; Glanzman et al., 1989; Glanzman et al., 1990). Thus, a single tail stimulus in the intact animal or a single (5 min) pulse of 5-HT in cell culture both produce short-term facilitation lasting minutes, whereas 4–5 tail stimuli or 4–5 pulses of 5-HT, applied over a period of 1.5–2 hr, elicit long-term facilitation lasting one day or more.

The increase in synaptic effectiveness with both short- and long-term synaptic facilitation results from an enhancement of transmitter release from the presynaptic (sensory) neuron (Castellucci and Kandel, 1976; Dale et al., 1988; Dale and Kandel, 1993). Whereas the short-term facilitation of the monosynaptic connection depends only on covalent modification of preexisting proteins, the long-term facilitation requires new protein and mRNA synthesis and is associated with the growth of new synaptic connections between the sensory and motor neurons (Montarolo et al., 1986; Castellucci et al., 1989; Glanzman et al., 1990). These structural changes, evident both in the intact animal and in dissociated cell culture, seem to reflect the most stable and persistent forms of memory (Bailey and Kandel, 1993).

As with behavioral learning, there is, for long-term facilitation, a brief early and labile consolidation period during which the long-term change is sensitive to disruption by inhibitors of protein and RNA synthesis (Castellucci et al., 1989; Montarolo et al., 1986). Long-term facilitation is no longer susceptible to disruption by inhibitors of protein synthesis 1 hr after the last of five pulses of 5-HT (2.5 hr after the onset of training) (Montarolo et al., 1986).

In dissociated cell culture, as in the intact ganglia, both short- and long-term facilitation can be induced by injection of cAMP into the presynaptic sensory neuron (Schacher et al., 1988; Scholz and Byrne, 1988), and both forms of facilitation are blocked by inhibitors of the cAMP-dependent protein kinase, protein kinase A (Ghirardi et al., 1992; Montarolo et al., 1992, Soc. Neurosci., abstract). With one pulse of 5-HT, which causes short-term facilitation, the activity of the catalytic subunit of protein kinase A is restricted to the cytoplasm and especially to the presynaptic terminal regions of the sensory neurons (Bacskai et al., 1993). By contrast, with repeated pulses of 5-HT, which give rise to long-term facilitation, the catalytic subunit translocates to the nucleus (Bacskai et al., 1993), where it appears to phosphorylate one or more cAMP response element–binding protein–related (CREB-related) transcription factors that activate cAMP-inducible genes (Kaang et al., 1993). Microinjection into the nucleus of a sensory neuron of an oligonucleotide containing the somatostatin cAMP response element (CRE) selectively blocks the long-term enhancement in synaptic strength induced by 5-HT without affecting the short-term process (Dash et al., 1990). Similarly, the transcription of a β-galactosidase reporter gene containing CRE elements upstream of the promoter (CRE–βGAL) is induced in the sensory neuron by five pulses of 5-HT (Kaang et al., 1993). Moreover, expression in Aplysia sensory neurons of a fusion gene (GAL4–CREB) encoding a *GAL4* DNA-binding domain and a mammalian CREB activation domain induces transcription of CRE–βGAL, and this induction requires a specific residue on CREB that serves as the site for phosphorylation by protein kinase A (Kaang et al., 1993).

We here attempt to follow further the sequence of steps whereby cAMP leads to the stable, self-perpetuating long-term process. The requirement for protein synthesis during a narrow time window, prior to onset of the stable long-term process, raises the following question. Are constitutively expressed regulatory proteins, such as CREB, coupled directly to effector genes essential for stable long-term facilitation, or are they coupled to these effectors by a cascade of one or more intervening regulatory genes? If intervening regulatory genes are involved, are these genes induced during the consolidation period, and if so, is their expression essential for long-term facilitation?

In a search for possible cAMP-dependent regulatory genes that might be interposed between constitutively expressed transcription factors and stable effector genes, we focused on the CCAAT enhancer–binding protein (C/EBP) transcription factors. The C/EBPs represent a family of transcription factors involved in terminal differentiation of a variety of cells, including adipocytes, enterocytes, hepatocytes, and monocytes. They are characterized by a bipartite (basic–leucine zipper) domain, which mediates DNA binding and homo- or heterodimerization (Landschulz et al., 1988; Landschulz et al., 1989; Vinson et al., 1989). Some members of the mammalian C/EBP family are responsive to cAMP (Metz and Ziff, 1991a) or bind to CREs in the regulatory sequences of gene transcription units (Park et al., 1990; Kageyama et al., 1991; Liu et al., 1991; Vallejo et al., 1993).

We therefore cloned and studied Aplysia C/EBP (ApC/EBP). We found that expression of ApC/EBP mRNA is not detectable by Northern blot analysis in the unstimulated central nervous system (CNS) of Aplysia. However, ApC/EBP is induced by 5-HT and cAMP during the consolidation phase of long-term facilitation. Induction of ApC/EBP mRNA by the action of 5-HT is rapid and transient, and it occurs in the presence of protein synthesis inhibitors. Moreover, blocking the synthesis of the ApC/EBP protein or the DNA binding activity of this protein selectively blocks long-term facilitation without affecting short-term facilitation. Thus, the induction of ApC/EBP seems to serve as part of a molecular switch activated during the consolidation period. This switch converts short-term synaptic plasticity, which does not depend on new protein synthesis, to long-term synaptic plasticity, which requires new protein synthesis during its consolidation phase. Part of the requirement for protein synthesis is the synthesis of ApC/EBP.

Results

Cloning of a cDNA Encoding ApC/EBP

In PC12 cells, C/EBPβ contributes to the cAMP-dependent regulation of *c-fos* transcription by acting on the serum responsive element, a 5′ regulatory region known to be essential for the induction by serum of *c-fos*. The serum responsive element contains several DNA-binding elements. One of these, the enhancer response element (ERE), is an 8-nucleotide sequence to which C/EBPβ binds (Metz and Ziff, 1991a, 1991b). We used a 17-mer oligonucleotide containing this ERE sequence to carry out DNA mobility shift assays and found that nuclear extracts from the Aplysia CNS contained ERE binding activity (Figure 3C). We therefore used ERE oligonucleotides to isolate cDNA clones encoding proteins that specifically bind this element. We screened an Aplysia cDNA expression library and isolated one clone, which showed a specific filter DNA binding activity for the ERE sequence but not for an unrelated oligonucleotide. The positive clone (clone 1) had an insert of 2.5 kb and contained a single open reading frame (ORF) in the frame with the β-galactosidase ORF. The first ATG codon in this frame, located 135 nucleotides 3′ to the fusion point, was tentatively assigned as the initiation codon. The predicted polypeptide was 286 amino acids long, encoded by the first 858 bp. The sequence continued with 1.57 kb of a putative 3′ untranslated region, ending with the poly(A) tail (Figure 1).

ApC/EBP Shows a Conserved Basic-Leucine Zipper Domain

In a database search, the putative protein encoded by Aplysia clone 1 showed the highest homology to the C/EBP

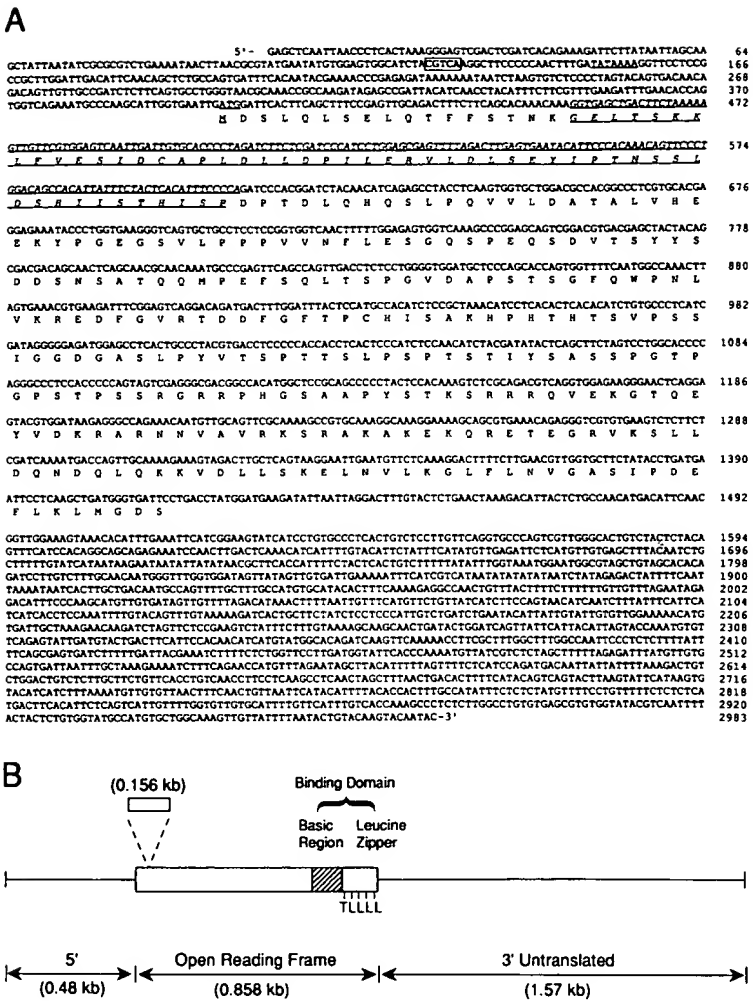


Figure 1. Nucleotide and Deduced Protein Sequences of ApC/EBP

(A) DNA and deduced amino acid sequence of the gene encoding ApC/EBP. The sequence represents both cDNA and genomic DNA. A TATAAA homology and the first ATG codon representing the putative translation start site are underlined. The CRE element is boxed. The two isolated clones (clones 1 and 2) differ in the 52 amino acid insert shown in underlined italics; the rest of the sequence, including the 3′ untranslated region, is identical. These sequences are deposited in GenBank under accession number U00994.

(B) A schematic representation showing the two ApC/EBP clones. The ORF coding region is shown as a boxed region. The hatched and stippled areas represent the basic region, followed by the leucine zipper domain.

family of transcription factors (Figure 2B). The predicted amino acid sequence of ApC/EBP clone 1 was 46% identical to its rat homolog C/EBPβ in the basic–leucine zipper domain, but it was only 25% identical in the remainder of the sequence (Figure 2A).

A search for potential phosphorylation sites revealed a common consensus sequence for phosphorylation within the leucine zipper domain by both the cAMP-dependent protein kinase and the Ca^{2+}-calmodulin-dependent kinase II (CamKII) (Figure 2A). This sequence is conserved in the Drosophila C/EBP (Figure 2B) and is distinct from the CamKII consensus phosphorylation site of mammalian C/EBPβ described by Wegner et al. (1992). In ApC/EBP, there are two potential phosphorylation sites for protein kinase C and two potential phosphorylation sites for tyrosine kinases. One of these lies in the basic region and is conserved in almost all cloned C/EBPs, including that of Drosophila. At positions 173 and 180, there are repeats of the mitogen-activated protein (MAP) kinase consensus site. The former mitogen-activated protein kinase site is conserved in both the rat and human C/EBPβs, and the rat C/EBPβ is phosphorylated in vivo by mitogen-activated protein kinase in response to activated *ras* (Nakajima et al., 1993). Finally, there are five consensus sites for casein kinase II (Figure 2A). The functional significance of these consensus sites for phosphorylation needs further investigation.

There Is a CRE Element in the 5′ Untranslated Region of ApC/EBP

To investigate whether the transcription of ApC/EBP might be regulated by CRE-binding proteins, we attempted to characterize the promoter region of ApC/EBP by cloning genomic ApC/EBP. From an Aplysia CNS genomic library, we isolated three genomic clones and found them to be identical using restriction analysis. The sequence analysis of one of those clones (clone 2) showed an ORF identical to that found for the cDNA clone, except that an in-frame coding insert of 156 bp was present after the eighteenth amino acid (see Figure 1). Canonical splicing junctions surround the 156 bp insert, suggesting that clone 1 is generated from clone 2 by alternative splicing. The 3′ untranslated region was entirely identical to that of cDNA clone 1. The more 5′ ATG codon, corresponding to the putative initiation codon, matched that found previously in the cDNA clone.

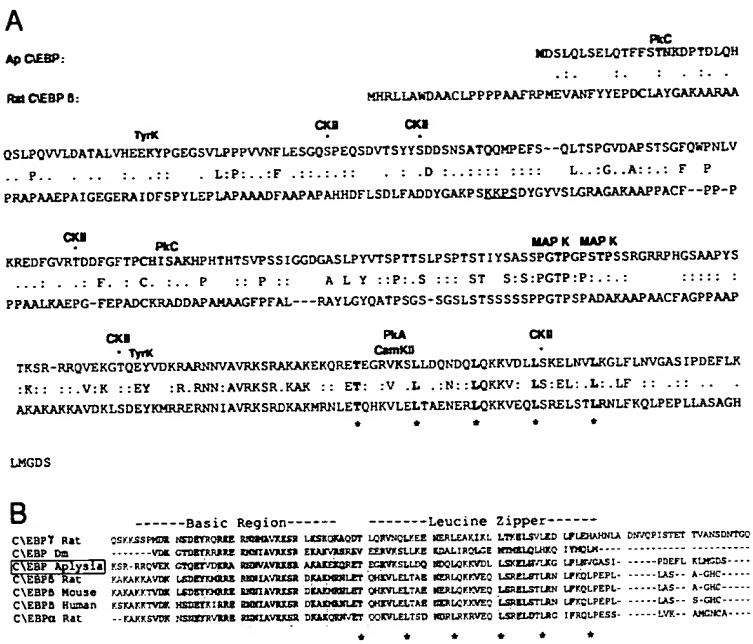


Figure 2. Comparison between ApC/EBP and Other C/EBP Family Members

(A) Amino acid alignment between ApC/EBP and rat C/EBPβ. Identical amino acids are repeated in the midline, and conserved residues are marked by colon or an ellipsis. Consensus sequences for putative phosphorylation acceptor sites are in a stippled box.

(B) Multiple amino acid alignment of the basic–leucine zipper domain of ApC/EBP and other C/EBP family members. Identical and similar amino acid residues are stippled, using Intelligenetics GeneWorks *2.1*. The asterisks mark the hydrophobic residues of the leucine zipper domain.

In the 5′ untranslated region of the genomic DNA sequence, we found a canonical TATAAA box homology sequence overlapping the TFIID/TATA-binding protein consensus sequence, occurring approximately 250 bp upstream of the putative initiation codon. We also found a putative catabolite gene activator protein site consensus sequence 27 bp downstream from the TATA homology. A computer search for binding sites of transcription factors revealed a putative nonpalindromic CRE site (Aplysia CRE [ApCRE]) 19 bp upstream of the TATA box, consistent with the possibility that ApC/EBP could be transactivated by a CREB-like protein.

ApC/EBP Binds to the ERE and to the Rous Sarcoma Virus Long Terminal Repeat Sequences

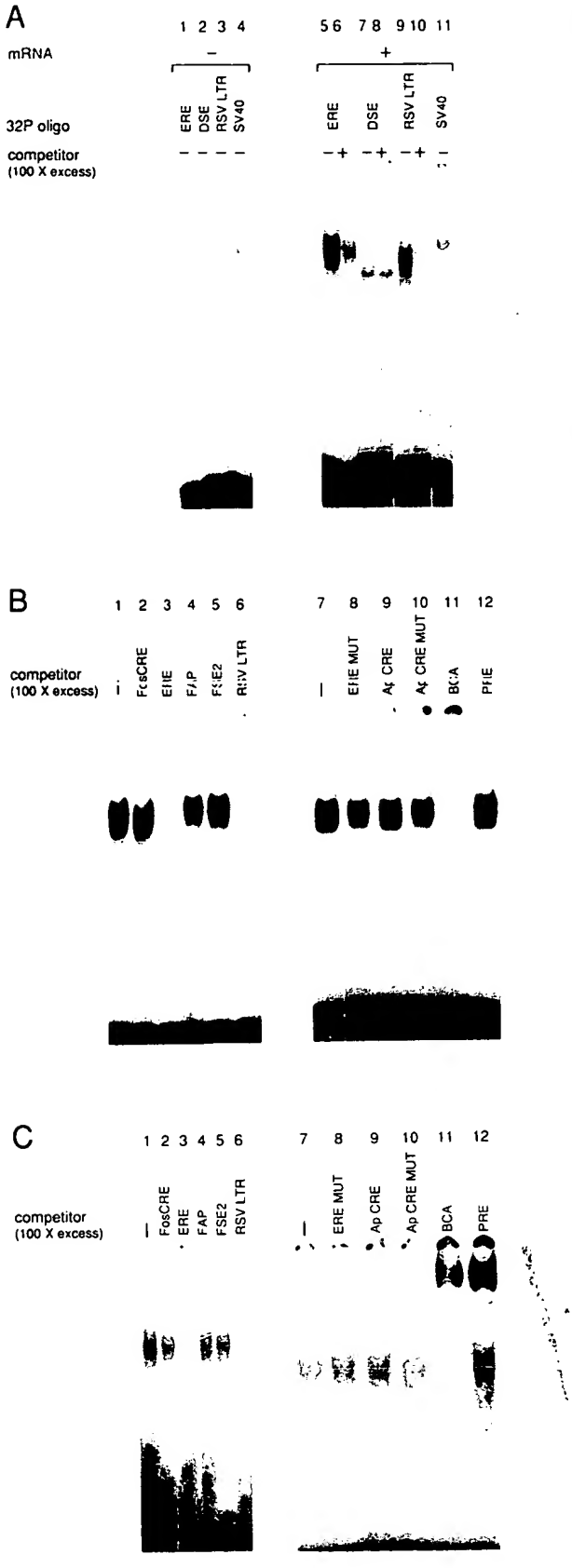
To characterize the DNA binding specificity of ApC/EBP, we tested seven different DNA-binding elements that are specific for a number of DNA-binding proteins (see Experimental Procedures; Figure 3). These included three DNA-binding sites specific for C/EBPs (ERE, Rous sarcoma virus long terminal repeat [RSV-LTR]), and SV40) and four DNA-binding sites (dyad symmetry element [DSE], *fos*–CRE, c-*fos* AP-1 site, and fat-specific element 2) recognized by unrelated transcription factors.

Using DNA mobility shift assays with in vitro translated ApC/EBP clone 1, we found specific binding of ApC/EBP to both C/EBP-binding sites, ERE, and RSV-LTR, but not to the DSE or the SV40 oligonucleotides (Figure 3A). To characterize better this binding specificity, we next carried out DNA binding competition studies using the ApC/EBP–ERE complex and various competitors (Figures 3B and 3C). Both the in vitro translated ApC/EBP (Figure 3B) and Aplysia CNS extracts (Figure 3C) bound the ERE ^{32}P-end-labeled oligonucleotide with a similar specificity. This binding was specifically competed only by C/EBP-specific DNA-binding sites (ERE and RSV-LTR), while none of the oligonucleotides (*fos*–CRE, c-*fos* AP-1 site, fat-specific element 2), containing binding elements for unrelated transcription factors, competed for ApC/EBP–ERE binding. In vitro translation of the ApC/EBP genomic clone yielded a protein showing the same oligonucleotide binding specificity (data not shown).

Moreover, we generated a polyclonal rabbit antiserum (BCA) against a bacterially expressed recombinant ApC/EBP protein. The antiserum BCA reacted specifically in western immunoblots with the bacterial recombinant ApC/EBP protein. The antiserum also immunoprecipitated ApC/EBP synthesized in vitro, as well as protein(s) with similar molecular weight from metabolically labeled Aplysia CNS (Figures 5A–5C). When BCA antiserum was added to DNA binding assays with ^{32}P-labeled ERE, carried out either with in vitro translated ApC/EBP or with Aplysia CNS extract, the oligonucleotide–protein interaction was disrupted selectively (Figures 3B and 3C). These data showed that ERE binding activity found in Aplysia CNS extracts is indeed due to the action of ApC/EBP.

ApC/EBP Is Induced by 5-HT as an Immediate-Early Gene during the Consolidation Phase

Tail stimuli that produce sensitization of the gill-withdrawal reflex lead to the activation of three classes of facilitating neurons. The most important of these classes uses 5-HT as its transmitter (Glanzman et al., 1989). Application of 5-HT to sensory and motor neurons in the intact ganglion or in dissociated cell culture leads to facilitation of the sensory motor neuron synapses. A single pulse of 5-HT produces a transient facilitation lasting minutes. By contrast, four or five pulses of 5-HT over a period of 1.5 hr produce facilitation that lasts more than one day. To determine the role of ApC/EBP in facilitation, we therefore



asked the following question: do repeated pulses of 5-HT affect the expression of ApC/EBP?

To investigate the expression of ApC/EBP in Aplysia neurons, we determined the relative concentrations of mRNA for ApC/EBP in untreated Aplysia CNS and in CNS exposed to 5-HT for 2 hr. Using Northern blot analysis, we found that the ApC/EBP mRNA was undetectable in the CNS from untreated animals, but that it was induced by 5-HT (Figure 4A). ApC/EBP mRNA was also induced when the intracellular concentration of cAMP was increased by using a cAMP analog in the presence of the phosphodiesterase inhibitor isobutyl methylxanthine or of forskolin in the absence or presence of isobutyl methylxanthine (Figure 4A).

To determine whether ApC/EBP is an early response gene that is activated directly by constitutively expressed transcription factors such as CREB, we examined the effects of the protein synthesis inhibitors anisomycin or emetine on the induction of ApC/EBP mRNA. In the presence

Figure 3. Binding Specificity of ApC/EBP

To characterize the binding specificity of ApC/EBP, we analyzed the binding activity of the following elements. Three oligonucleotides contained transcription factor–binding sites identified in *fos* serum responsive element or CRE enhancers (Metz and Ziff, 1991b): the DSE, the c-*fos* AP-1 (FAP) site, and the c-*fos* CRE (*fos*–CRE) (Gilman et al., 1986). As the DSE consensus sequence largely overlaps the ERE-binding site, it provided a sensitive test of binding specificity (Metz and Ziff, 1991b). In addition, we tested an oligonucleotide derived from the promoter of the RSV-LTR, which has been shown to be activated when bound to a CamKII-phosphorylated C/EBPβ (Kapiloff et al., 1991; Wegner et al., 1992), and we tested the core element of SV40 enhancer, which contains a C/EBP-binding consensus but is not activated by CamKII-activated C/EBPβ (Kapiloff et al., 1991; Wegner et al. 1992). Finally, we also tested the fat-specific element 2 (FSE2) of the *ap-2* gene, which is recognized by *fos/jun* family members (Distel et al., 1987).

(A) In vitro synthesized ApC/EBP clone 1 binding activity. ApC/EBP obtained by RNA that was in vitro translated in reticulocyte lysate (lanes 5–11) or control reactions, performed in the absence of RNA (lanes 1–4), were assayed by DNA mobility shift assay with ^{32}P-end-labeled ERE (lanes 1, 5, and 6), DSE (lanes 2, 7, and 8), RSV-LTR (lanes 3, 9, and 10), or SV40 (lanes 4 and 11). Competition reactions (lanes 6, 8, and 10) were performed adding the corresponding, nonradiolabeled oligonucleotide at 100 molar excess. Note that the bands generated in the DSE or SV40 reactions are nonspecific because the same bands are present in the control reaction performed in the absence of mRNA and, therefore, they likely reflect interactions with proteins present in the reticulocyte lysate.

(B) Competition for ERE binding of in vitro synthetized ApC/EBP. ^{32}P-end-labeled ERE oligonucleotides were incubated with in vitro translated ApC/EBP in the absence or presence of a 100 molar excess of the indicated competitor oligonucleotides and electrophoresed. In addition, to test the reactivity of the anti-C/EBP antiserum (BCA) or of the preimmune serum (PRE) against the ApC/EBP–ERE binding, the in vitro generated protein was preincubated with 1:50 diluted BCA or PRE for 15 min, at the end of which ^{32}P-labeled ERE was added for an additional 30 min and then electrophoresed.

(C) Competition for ERE binding of Aplysia nuclear extracts. ^{32}P-end-labeled ERE oligonucleotides were incubated with Aplysia nuclear extracts obtained from the total CNS, which had been dissected and incubated in medium at 18°C for 7 hr to induce high expression of ApC/EBP. The concentrations of cold oligonucleotides or BCA and preimmune sera (PRE) used for competition were the same as described in (B).

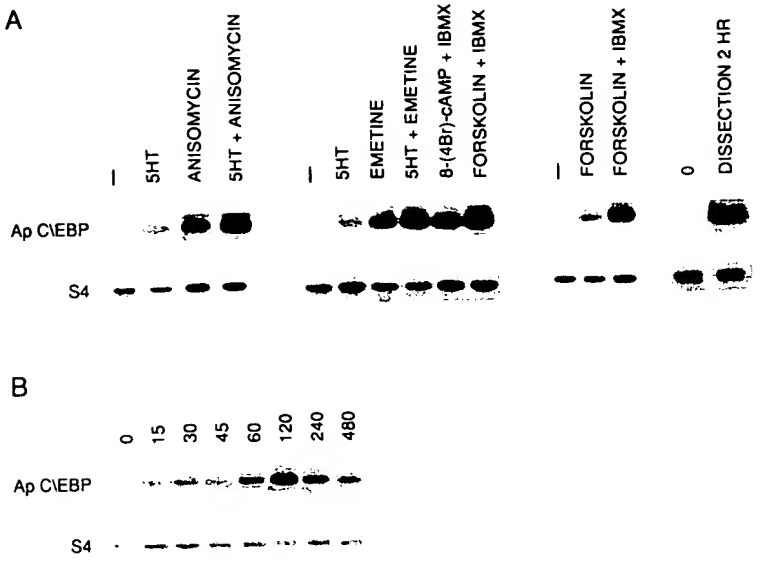


Figure 4. Induction of ApC/EBP mRNA

(A) ApC/EBP mRNA expression in CNS of untreated Aplysia, of Aplysia treated in vivo with the indicated drugs for 2 hr at 18°C, or dissected without treatment and kept at 18°C in culture medium. Shown here are four independent experiments, in which 10 μg of total RNA extracted from the CNSs of untreated (minus sign) or treated Aplysia, as indicated, were electrophoresed, blotted, and hybridized with ^{32}P-labeled ApC/EBP (top) or S4 (bottom) probes. The latter encodes the Aplysia homolog of S4 ribosomal protein (Thomas et al., 1987), which is constitutively expressed and used as a loading control. Zero indicates RNA extracted immediately after dissection of Aplysia CNS. The 2 hr dissection represents RNA extracted from Aplysia CNS, dissected, and incubated in culture medium for 2 hr at 18°C. IBMX, isobutyl methylxanthine.

(B) Time course of ApC/EBP mRNA induction following 5-HT treatment. Times of treatment, in minutes, are indicated. Five micrograms of the total RNA extracted from the total CNS of in vivo treated Aplysia were analyzed as described in (A).

of these inhibitors, the induction of ApC/EBP by 5-HT was increased (Figure 4A). These data define ApC/EBP as an immediate-early or primary response gene. These genes, many of which are transcription factors, are poorly expressed in unstimulated cells but are induced rapidly and transiently in response to various ligands, even in the presence of inhibitors of protein synthesis (reviewed by Herschman, 1991).

Protein synthesis inhibitors by themselves produced an induction of the ApC/EBP mRNA larger than that produced by 5-HT (Figure 4A). However, all of the Northern blot experiments were carried out with the whole CNS of Aplysia, in which only some cells respond to cAMP or to 5-HT, whereas most cells are likely to show an effect of anisomycin on the mRNA for ApC/EBP. We have therefore quantified by densitometric analysis in two experiments the increase of ApC/EBP mRNA in response to 5-HT, protein synthesis inhibitors, or both. In both experiments, we found that the effects of 5-HT and protein synthesis inhibitors were not simply additive. Giving a unitary value to the induction produced by 5-HT alone, anisomycin showed an induction of 2-fold, and 5-HT and anisomycin together resulted in an induction of 7.3-fold. Emetine produced an increase of 6.8-fold compared with that produced by 5-HT alone. By contrast, 5-HT and emetine together produced an induction of 14.4-fold. Thus, the superinduction due to protein synthesis inhibitors suggests that the induction of ApC/EBP transcription by 5-HT is regulated by constitutively expressed proteins. Moreover, as we show below, this induction is rapid, consistent with C/EBP being a primary response gene.

Moreover, as has been found frequently with immediate-early genes, ApC/EBP was rapidly and dramatically induced following tissue dissection (Figure 4A).

ApC/EBP Is Induced Rapidly by 5-HT during the Consolidation Period

Analysis of the time course of the induction of ApC/EBP mRNA following exposure to 5-HT in vivo showed that the ApC/EBP mRNA is induced rapidly. Within 15 min after 5-HT is applied, an increase in the level of mRNA is evident. The mRNA reached its peak level of expression 2 hr after the application of 5-HT and began to decrease 4 hr after the onset of treatment (Figure 4B).

In addition to examining the concentration of the mRNA, we examined the induction of the protein in individual cells in culture in the presence and absence of 5-HT. Toward this end, we used the BCA antibody to detect the expression of ApC/EBP protein in cultured Aplysia sensory neurons. The expression of ApC/EBP protein was not detectable in untreated sensory neurons. After exposure to five pulses of 5-HT, there was clear nuclear staining in cells incubated with anti-ApC/EBP antiserum (BCA), but no staining in cells incubated with preimmune serum (PRE; Figure 5D). ApC/EBP is therefore induced during the consolidation period, the time window in which RNA and protein synthesis are required for converting short- to long-term facilitation. This raised the question: is C/EBP required in the sensory neurons to achieve long-term facilitation?

Injection of Oligonucleotides Containing the ERE Blocks Long-Term Facilitation

To determine whether ApC/EBP was an immediate-early gene required for long-term facilitation, we attempted to interfere with the binding of the ApC/EBP protein to its DNA-binding element on regulatory regions of target genes. To compete for the binding activity of the ApC/EBP to its target sequence, we adopted the experimental

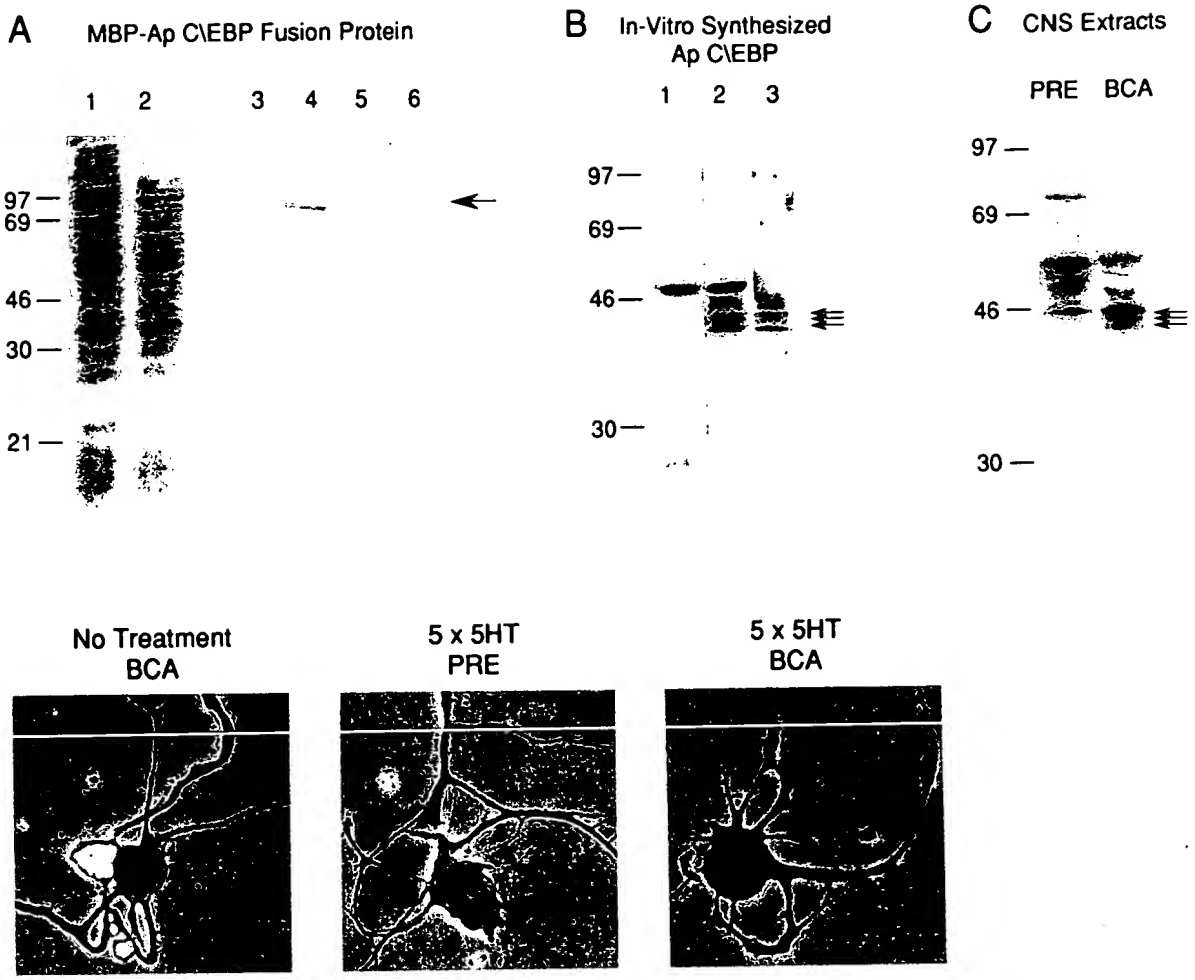


Figure 5. ApC/EBP Protein Induction in 5-HT-Treated Sensory Neurons Detected by Anti-ApC/EBP Rabbit Antiserum

(A–C) Antiserum BCA recognizes specifically the maltose binding protein–ApC/EBP fusion protein, and it immunoprecipitates in vitro synthesized ApC/EBP and protein(s) from Aplysia CNS extracts with similar molecular weight.

(A) Lanes 1 and 2, Coomassie blue staining of 10% SDS–PAGE. Lane 1 contains noninduced ApC/EBP–*malE* bacterial extracts, and lane 2 contains IPTG-induced ApC/EBP–*malE* bacterial extracts. Part (one-tenth) of the same extracts (lane 3, noninduced, and lanes 4–6, IPTG-induced) were electrophoresed under the same conditions, transferred to nitrocellulose, and probed with BCA antiserum (lanes 3 and 4), with preimmune serum collected from the same rabbit (lane 5), or with an unrelated rabbit antiserum raised against the Aplysia Y box–binding protein (lane 6) (Paul Skehel, personal communication). The fusion protein maltose binding protein–ApC/EBP is indicated by an arrow.

(B) [^{35}S]methionine-labeled in vitro translated ApC/EBP was analyzed by 10% SDS–PAGE, and then it was autoradiographed. Lane 1 contained a control reaction and had no ApC/EBP mRNA. Lane 2 contained ApC/EBP that was in vitro transcribed and translated. Lane 3 showed immunoprecipitation with BCA antiserum of in vitro translated ApC/EBP. Arrows indicate the bands corresponding to the ApC/EBP protein(s).

(C) Autoradiography of 10% SDS–PAGE illustrates immunoprecipitations with PRE or anti-ApC/EBP (BCA) antiserum of Aplysia extracts after metabolic labeling with [^{35}S]methionine and [^{32}P]orthophosphate. Arrows indicate specific bands immunoprecipitated by BCA antiserum. Bars alongside autoradiographs indicate positions of relative molecular mass markers. PRE, preimmune serum.

(D) ApC/EBP protein is induced in cultured sensory neurons upon exposure to 5-HT. Cultured Aplysia sensory neurons, nonexposed or exposed to 5 pulses of 5-HT (5× 5-HT), were fixed and stained with BCA or PRE rabbit (anti)sera and gold-conjugated goat anti-rabbit antibody.

approach of Dash et al. (1990), who injected somatostatin CRE oligonucleotide to compete for endogenous CRE binding activity and showed that this selectively blocked long-term facilitation. After measuring the strength of the evoked excitatory postsynaptic potential (EPSP), we injected into sensory cells in culture either ERE, ApCRE, or control oligonucleotides. After injection, the cultures received five pulses of 5-HT. The degree of facilitation was monitored 24 hr later (Figure 6).

Cells injected with ERE oligonucleotides showed no increase in the strength of their synaptic connections 24 hr after treatment with 5-HT (−10.4% ± 10.7, n = 14). Their connections were comparable to control cells that were not exposed to 5-HT (−10.3% ± 4.2, n = 7) and were significantly different from uninjected cultures exposed to 5-HT (+73.9% ± 15.9, n = 14, $p < 0.01$, Newman Keuls' multiple range test; Figures 6B and 6C). By contrast, cells injected with an ERE oligonucleotide mutated at two bases